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journal homepage: www.elsevier.com/locate/bbabbioIs coproporphyrin III a copper-acquisition compound in *Paracoccus denitrificans*?Jani Anttila^a, Petri Heinonen^b, Timo Nenonen^a, Andrea Pino^a, Hideo Iwaï^c, Eeva Kauppi^d,
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ABSTRACT

Paracoccus denitrificans is a soil bacterium which can respire aerobically and also denitrify if oxygen is absent. Both processes are highly dependent on copper enzymes and copper is therefore likely to be an essential trace element for the bacterium. If copper is not easily available, a copper-acquisition mechanism would be highly beneficial. In this paper, we have addressed the question of whether *Paracoccus* secretes a copper-acquisition compound functionally analogous to that found in some methanotrophs. Bacteria were grown both in copper-containing and copper-deficient denitrification media, cells were removed by centrifugation and the supernatant was analysed using chromatography and spectroscopy. Bacterial growth yield in the absence of copper was 70–80% of that in the copper-containing medium. A notable difference between the two culture conditions was that spent copper-deficient medium was pigmented, whereas the copper-containing medium was not. Spectrophotometry indicated that a red compound with an absorption maximum at 405 nm was produced under copper-limited conditions. In addition to the strong 405 nm maximum, the visible spectrum of the purified red molecule had weaker maxima at 535 nm and 570 nm, features typical of metallated tetrapyrroles. Mass spectrometry showed that the purified pigment had a molecular mass of 716.18. Moreover, the fine structure of the mass spectrum suggested the presence of zinc and was consistent with the chemical formula of $C_{36}H_{36}N_4O_8Zn$. The presence of zinc was also demonstrated using inductively coupled plasma atomic emission spectroscopy. Fragmentation analysis with mass spectrometry showed the release of consecutive 59 Da fragments, assignable to four $-CH_2-COOH$ moieties. Thin layer chromatography as well as NMR analysis of the C-13/N-15 labelled red pigment suggested that it is predominantly zinc coproporphyrin III with a minor fraction of metal-free coproporphyrin III. We propose that in a copper-poor environment *P. denitrificans* secretes coproporphyrin III for copper chelation and subsequent uptake of the bound copper into the cell. Consistent with this idea, cell yields of copper-deficient cultures grown in the presence of 1 μM copper-coproporphyrin III were 90–95% of the yields of cultures grown in the normal copper-containing media. Coproporphyrin III may work as a copper-acquisition compound in *P. denitrificans*.

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1. Introduction

Copper ion constitutes a key part in the active sites of many redox enzymes. In particular, several crucial respiratory and denitrification enzymes (heme-copper cytochrome oxidases, nitrous oxide reductase and one of two nitrite reductases) are copper proteins [1–3]. Since both respiration and denitrification are major energy transducing pathways responsible for ATP generation, their proper functioning is paramount for the organism. Copper is a trace element and its availability may sometimes be growth limiting. We envision that under copper poor conditions, a molecular system capable of scavenging

copper and delivering it to the cell would give a microbe a significant advantage over other organisms.

So far only one such copper-acquisition molecule has been reported: methanobactin in two methanotrophic soil bacteria. In a copperless culture medium, *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b have been shown to secrete a copper-specific chelator (a chalkophore) known as methanobactin [4,5]. It appears to provide copper for an essential copper-containing enzyme methane mono-oxygenase. The object of our study is *Paracoccus denitrificans*, a soil bacterium capable of living under a range of conditions [6]. In the presence of oxygen, it can respire using an electron transfer chain closely related to the mitochondrial respiratory chain [2,7]. Under anaerobic conditions and in the presence of nitrate, *P. denitrificans* denitrifies. The efficient operation of both respiration and denitrification depends strongly on copper

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enzymes and it is reasonable to expect that *P. denitrificans* could have evolved ways to ensure copper acquisition in case its environment is copper poor. In this study, we report that upon copper limitation, *Paracoccus* secretes a red pigment which we identify as zinc coproporphyrin III. We propose that secreted coproporphyrin III can act as a copper-acquisition compound in *P. denitrificans*.

2. Materials and methods

2.1. Culturing the bacteria and collecting the spent medium

P. denitrificans 1657 [8] was grown under denitrifying conditions either on a succinate minimal medium [7] containing 10 g/l KNO₃ or on similar glucose/nitrate medium in which succinate was replaced with 0.4% (w/v) glucose. Both media were supplemented with 100 mg/l yeast extract (Difco) and with a salt solution [7] giving final concentrations of 0.1 mM Ca²⁺, 90 μM Fe³⁺, 50 μM Mn²⁺, 25 μM Zn²⁺, 10 μM Co²⁺, 5 μM H₃BO₃, and 10 μM MoO₄²⁻ and, when present in control experiments, 5 μM Cu²⁺. pH was adjusted to 7. 2–3 l cultures were inoculated with 100–150 ml of overnight starter cultures grown in Luria–Bertani medium, incubated for 4–6 days at 30 °C without shaking until they reached early stationary phase (Klett number about 500 using a 660 nm filter). In some cases, the salt solution was first added to the well-grown starter cultures which were used within a few minutes to inoculate the culture bottles containing the glucose medium. This inoculation protocol resulted in overproduction of the red pigment. In some experiments, the amount of zinc in the culture medium was lowered to 1 μM but formation of zinc coproporphyrin could still be observed in copperless media. When studying the effect of copper coproporphyrin on growth, 0.5 μM or 1 μM Cu-coproporphyrin III was included in the copperless growth media. When producing samples labeled with ¹³C and ¹⁵N, the growth medium contained 0.4% ¹³C₆ glucose and 1 g/l ¹⁵NH₄Cl instead of the corresponding normal reagents. The cells were spun down and the cell pellets were weighed for assessing the growth yield. The spent culture medium was filtered through a filter paper and then loaded on an Oasis HLB solid extraction cartridge (waters). A strongly red fraction was eluted with methanol according to the manufacturer's instructions. This eluted material was stored in dark at –20 °C and was purified further at room temperature with either protocol A or B.

2.2. Purification protocol A

The red fraction was applied to a column of immobilised copper resin (Chelating Sepharose Fast Flow (loaded with Cu²⁺). The copper column (GE Healthcare XK 26/10) had been equilibrated with 20 mM Tris–Cl, 0.5 M NaCl and 0.1 mM imidazole, pH 7.6, and was eluted with 50 mM imidazole in the same buffer. Absorbance at 280 nm or 405 nm was monitored. Reddish fractions eluted either immediately after the wash-through (a major fraction that interacted only weakly with the copper column), or after the imidazole gradient had reached 47 mM [imidazole] comprising about 10% of the total red pigment. A copper determination [9] as well as a type 2 copper signal in EPR spectroscopy at 10–12 K showed that both fractions contain copper. The major and minor fractions were concentrated with an Oasis HLB cartridge and eluted with methanol. Optical spectra (Fig. 2) were measured in 50% methanol with a Shimadzu UV 2401 PC spectrophotometer. Fluorescence spectra from spent stationary phase culture media diluted at 1:100 with 20 mM Tris–Cl, pH 7.6 were recorded with Fluoromax-4 Spectrofluorimeter (Horiba Jobin Yvon, NJ, USA), using excitation at 405 nm.

Some preparations were concentrated with Oasis Wax cartridges and analysed with a Bruker Esquire ESI mass spectrometer equipped with a nano-HPLC apparatus for direct injection to the mass spectrometer. A PepMap C-18 reverse phase column with particle and bore sizes of 3 μm and 100 Å was equilibrated with buffer A

(10 mM ammonium acetate, pH 7, 5% methanol) and eluted with buffer B (10 mM ammonium acetate, pH 7, 80% methanol). Standard HPLC buffers containing 10 mM ammonium acetate, pH 7, 5% acetonitrile, 0.05% trifluoroacetic acid (A) and 10 mM ammonium acetate, pH 7, 80% acetonitrile, 0.04% trifluoroacetic acid could also be used. Both major and minor fractions yielded similar chromatograms in which the main species absorbing at 400 nm elutes with 80% methanol. The mass of this species was 715.3 Da in both major and minor fractions. In some preparations, a mass of 716.3 Da was also observed.

For mass spectrometry with a Synapt G2 HDMS Q-TOF instrument (waters), the methanolic solvent was exchanged for 95% acetonitrile–0.1% trifluoroacetic acid using Zip-Tips (Millipore). The mass spectrometer was calibrated with Glu fibrinopeptide B fragment (Glu FiB, Sigma) over a mass range of 50 to 1200 Da. Glu FiB m/z of 785.84265 was used as the lock mass. The observed high resolution masses of the pigment fractions near m/z = 715 were 715.1939 and 716.1939.

2.3. Purification protocol B

Samples purified with this protocol had not been exposed to copper, and were used for metal determinations with inductively coupled atomic emission spectrometry, for high resolution mass spectrometry and for NMR analyses.

0.5–0.8 ml of the total methanolic fraction from the cultures was centrifuged briefly, diluted with water (1:5) and applied to a column containing 8 ml of Source 15RPC resin (GE Healthcare) equilibrated with 10 mM ammonium acetate, 5% methanol. The column was developed with 10 mM ammonium acetate, 90% methanol using an Äkta FPLC instrument. The red pigment eluted at 36% methanol. Fractions with the highest absorbance at 405 nm were concentrated by lyophilisation with a Hetovac.

Most samples for NMR and high resolution mass spectrometry were purified further with a reverse phase column (Inertsil HPLC Peptides C18, 10 mm × 250 mm, GL Sciences) using an Äkta Purifier apparatus and 10 mM ammonium acetate (pH 7) buffers with 5% (A) and 90% (B) acetonitrile. The fractions with the highest absorption at 405 nm were pooled and lyophilized. As an alternative for this HPLC step, an ion exchange step using a 1 ml HiTrap DEAE-Capto could also be used. The column was equilibrated with 20 mM Tris–Cl, pH 7.6, 20% methanol and eluted with 20 mM Tris–Cl, pH 7.4, 30% methanol, 1.75 M NaCl. The red pigment eluting near the maximum salt concentration was desalted with an Oasis HLB cartridge and lyophilized for further experiments.

2.4. Metal analyses

Copper contents of chromatographic fractions with a visible absorption spectrum were determined using a colorimetric method [9]. The contents of copper, iron, zinc and sulfur were determined with inductively coupled plasma atomic emission spectrometry (ICP-AES) at VTT Expert Services, Espoo, Finland. Prior to the measurement, dried desalted samples were dissolved in 5% nitric acid. Only zinc was present in the fractions with an optical spectrum similar to P in Fig. 2. These fractions were used in the NMR and thin layer chromatographic analyses (see below).

2.5. Mass spectrometry and NMR spectroscopy

High resolution mass spectra were recorded with a Bruker Micro-TOF mass spectrometer using electrospray ionization in positive ion mode. The samples were dissolved in 80% HPLC-grade acetonitrile. A Bruker Esquire mass spectrometer equipped with an electrospray ion source was used in the fragmentation analysis of the red pigment.

The NMR spectra were measured from $^{15}\text{N}/^{13}\text{C}$ labelled samples using a Varian Unity INOVA NMR spectrometer operating at 500 MHz for protons. The lyophilized samples were dissolved in 0.6 ml of DMSO- d_6 which was also used as a chemical shift reference (2.49 ppm for ^1H and 39.5 ppm for ^{13}C). All spectra were recorded at 30 °C. The processing of the spectra was performed with Varian VNMR and Spinworks 2.5 programs. Assignment of the structure was based on ^1H , ^1H with ^{13}C decoupling, ^1H – ^{13}C –heteronuclear single quantum coherence (HSQC), ^1H – ^{13}C –heteronuclear multiple bond correlation (HMBC), ^1H – ^{13}C –HSQC-TOXY and ^1H – ^{15}N -HMBC experiments. In ^1H – ^{13}C -HSQC and ^1H with ^{13}C decoupling experiments, 0.5 mg/ml (final concentration) zinc acetate was added to the sample.

2.6. Thin-layer chromatography

Thin-layer chromatography was carried out by modifying the paper chromatographic method of Eriksen [10] and Aziz et al. [11] as follows: A 10 cm × 10 cm Silica gel F254 (Merck) plate was developed with 5:2 2,6-lutidine/water in an ammoniacal atmosphere. Coproporphyrins I and III were purchased from Livchem, Germany. The metallated standards were prepared by mixing 0.1 mg of coproporphyrin with 0.11 ml of 50 mM ammonium acetate–50% methanol, 5 μl 25% (w/w) ammonia and 15 μl of 10 mM zinc acetate. Copper-coproporphyrin III was prepared similarly, but using a slightly substoichiometric amount of copper relative to the porphyrin. To a 5 μl sample of 0.8 mM red pigment (purified from bacterial culture media) in 80% methanol, 1 μl of 25% ammonia was added. For chromatography, 1 μl aliquots of the standards and the sample were applied to the thin-layer plate. The migration of the compounds during and after the chromatography was observed visually, making use of their strong purple-red colour.

3. Results

The growth curves in Fig. 1A show that *P. denitrificans* grew relatively well in the copperless media. The logarithmic growth phase lasted about two days. In fact, it seems that the control cells had first a one day lag phase, probably because 5 μM Cu^{2+} in the growth medium is slightly toxic at a low cell density. The control culture however started growing more vigorously after the first day. A notable visual difference between the copperless and copper-containing cultures was that the copperless cultures turn light red, whereas the control cultures did not. This phenomenon was stronger in the glucose medium but it occurred also in the succinate medium. The final cell yield of the copper-poor cultures was 70–80% of the yield of control cells grown in the copper-containing media. Complementation of the copperless media with 1 μM copper coproporphyrin III resulted in a cell yield which was 90–95% of the yield in normal copper-containing media, indicating (i) that growth in the copper-poor media was copper-limited and (ii) that copper coproporphyrin could be used as a source of copper. Moreover, inclusion of Cu-coproporphyrin in the culture media partially inhibited the formation of the red pigment, identified below as the zinc coproporphyrin III complex.

The appearance of the red pigment was monitored by measuring the absorbance of the cell free medium at 405 nm when the cells grow (Fig. 1B). With the bacteria in the copperless medium, the concentration of the pigment reached a maximum in the early stationary growth phase. The control cells produced very little of the red molecule under the same conditions. We conclude that the presence of the red pigment correlated strongly with the absence of added copper. Furthermore, the appearance of the pigment was not restricted to denitrification conditions, as the pigment was also observed in aerobic succinate-grown copper-limited cultures (data not shown).

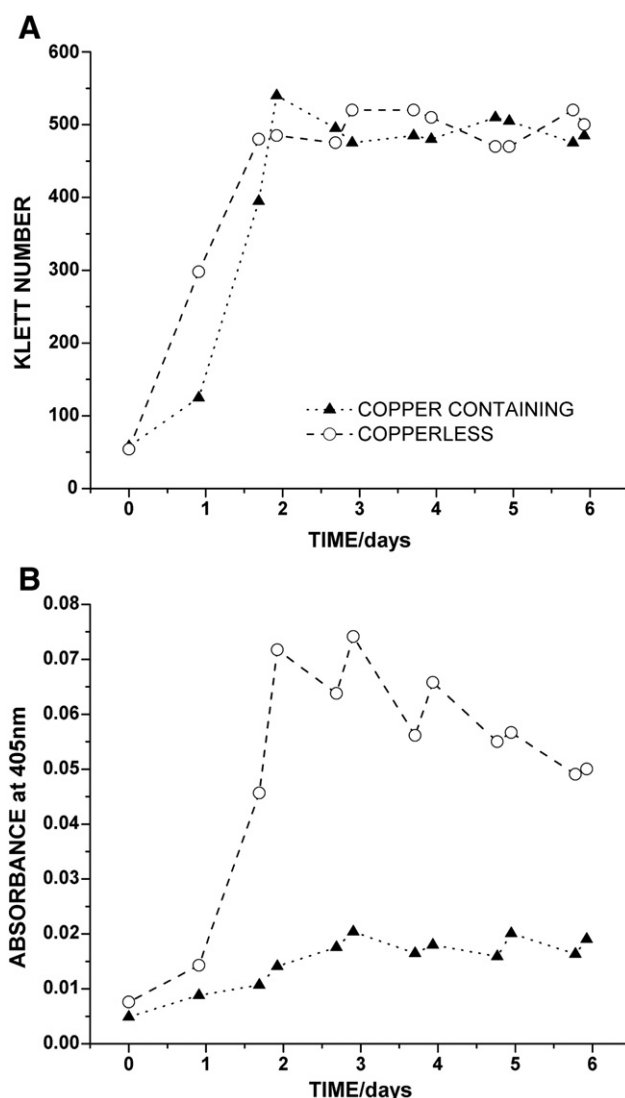


Fig. 1. A. Growth curves of *P. denitrificans* 1657 in the copper-containing and copperless media. The final cell yields in the absence and presence of copper were 9.7 and 12.1 g of wet cells/l medium. B. Production of the red pigment (as measured by the absorbance at 405 nm) during the growth of bacteria in absence of added copper. Filled and open symbols represent copper-containing and copper-deficient cultures, respectively.

3.1. Purification and analytical studies of zinc coproporphyrin III

We initially attempted to isolate the red pigment from the spent culture medium using an immobilized copper column (protocol A in Section 2.2 and Section 3.2). However, this protocol combined with further reverse phase HPLC steps invariably resulted in samples that did not give good NMR signals, probably because of residual paramagnetic copper in the samples. For this reason, we decided to omit the copper column and proceeded as follows: normal or ^{13}C - and ^{15}N -labelled cells were grown and the spent culture medium was concentrated using a solid phase extraction cartridge. The unlabeled or labeled red pigment was then purified using protocol B consisting of two reverse phase HPLC steps or one HPLC and one ion-exchange step (Section 2.3). The procedure yielded bright red material, the optical spectrum of which had a strong feature at 405 nm as well as minor features above 520 nm (spectrum P in Fig. 2). This material was used in the high-resolution mass spectrometry and 2D NMR experiments described below.

The mass spectrum of the red pigment (Fig. 3) purified using protocol B gave a high resolution mass of 716.1807 followed by five to six noticeable isotope peaks separated by 1 Da. These features

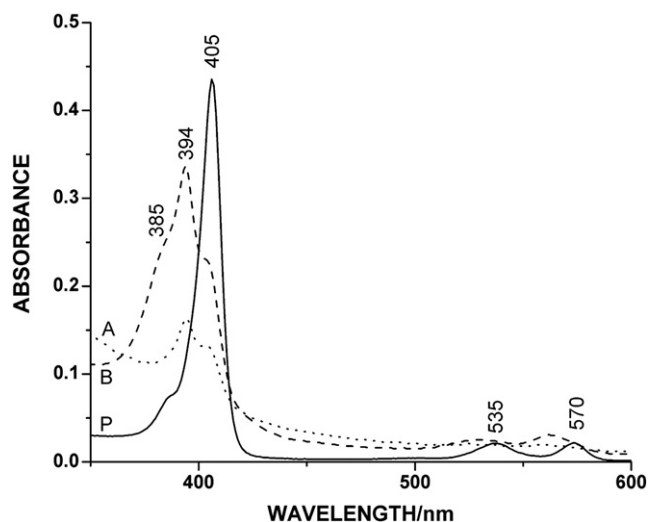


Fig. 2. Optical spectra of fractions containing the red pigment. A, Red pigment eluted from the immobilized copper column with imidazole. B, Red pigment which binds weakly to the copper column. P, Red pigment (zinc coproporphyrin III) purified with protocol B from the spent copperless culture medium of *P. denitrificans*. The spectrum of the spent medium is identical with spectrum P. The Soret maxima of zinc and copper coproporphyrins are at 405 and 394 nm, respectively. The spectra were measured in 50% methanol.

suggested the presence of zinc in the sample. The found mass was in line with the molecular formula of $C_{36}H_{36}N_4O_8^{64}Zn$, the formula of zinc coproporphyrins. Furthermore, the simulated isotope pattern for the given molecular formula (Fig. 3, bottom panel) matched well the observed isotope distribution (Fig. 3, upper panel). Also, the optical spectrum of the red pigment (Fig. 2) was very similar to the published spectrum of zinc coproporphyrin [12]. ICP-AES analysis of the protocol B purified red pigment showed that only zinc and no copper or iron were present in the sample. An ion trap MS/MS fragmentation analysis showed that four groups with $m/z = 59$ Da could be sequentially released (data not shown). These 59 Da fragments were likely to have arisen from the cleavages between C-2 and C-3 of each of the four propionate side chains of the tetrapyrrole.

3.2. Characterization of the red pigment purified using an immobilized copper column

As in protocol B, the culture supernatant was first concentrated and then applied to an immobilized copper column. The red pigment eluted in two separate fractions: a tightly-binding minor fraction A (10–15% of total) and a loosely-binding fraction B (85–90% of total) that was only slightly retarded during its way through the column. Fraction A bound strongly to the column, was eluted with 45–50 mM imidazole and appeared thus to have clear copper binding activity. Copper analysis showed however that both fractions contain copper, likely to have bound during the metal affinity chromatography. The optical spectra of the two fractions (spectra A and B in Fig. 2) were similar to each other and showed features typical of porphyrins, e.g. a strong Soret band near 400 nm, and weaker β and α bands above 520 and 560 nm, respectively (cf. [12,13]). The comparison of these spectra with the spectrum of the red pigment in the culture medium or purified using protocol B suggested that in A and B there was a spectral feature blue-shifted relative to the spectrum P of the purified red pigment. The Soret maximum in spectra A and B has shifted to 394 nm (with a shoulder at 405 nm), consistent with the reported γ – maximum of copper coproporphyrin [14]. Mass spectrometry showed that the major, loosely-bound fraction was dominated by the mass of 716.2 ± 0.1 Da, whereas the mass of 715.2 ± 0.1 Da was observed in the tightly-bound fraction.

Since these results together the NMR and thin-layer chromatographic (TLC) data below suggest that the molecule giving rise to spectrum P is zinc coproporphyrin III (Section 3.3), it is likely that the blue-shifted component in spectra A and B originated from copper coproporphyrin III. In particular, the measured high resolution mass of 715.2 fits the mass of ^{63}Cu coproporphyrin (and the mass of 716.2 Da corresponds to that of ^{64}Zn coproporphyrin). We conclude that copper coproporphyrin III was formed during the immobilized copper chromatography, demonstrating the copper binding capability of the red pigment. Moreover, the occurrence of this compound in the minor, tightly-binding fraction may reflect the presence of free coproporphyrin in the spent copperless growth medium. Consistent with this idea, the fluorescence spectrum of spent culture medium (with excitation at 405 nm) showed a minor emission maximum at 615 nm, which we

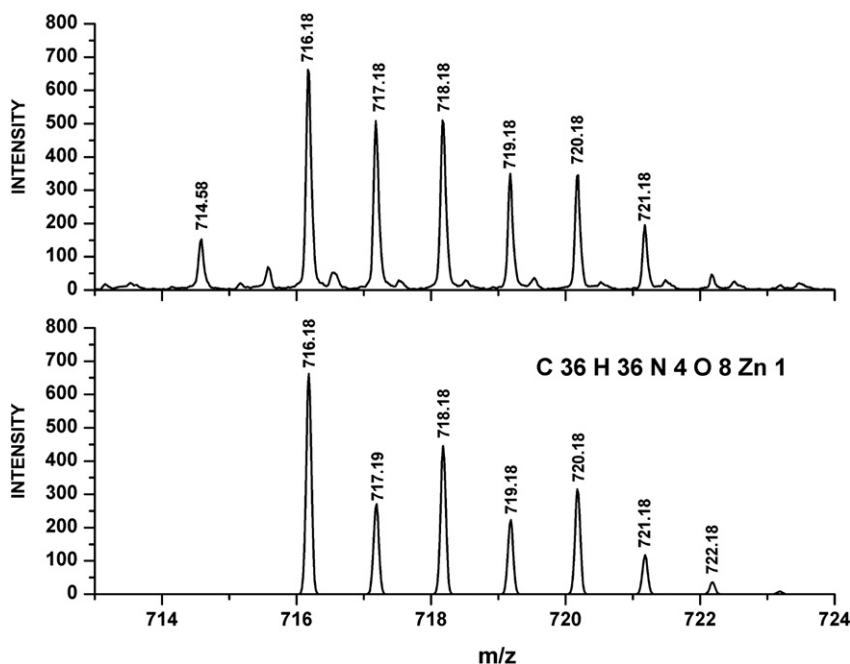


Fig. 3. Upper panel. High resolution mass spectra of the red pigment purified with protocol B. Bottom panel. A mass spectrum simulated using the formula of zinc coproporphyrin and the natural abundance ratios of the six stable isotopes of zinc. 716.1819 Da corresponds to the mass of ^{64}Zn coproporphyrin.

assign to the free coproporphyrin. The major emission maximum at 575 nm can be attributed to zinc coproporphyrin (cf. [12]).

3.3. Verification of the coproporphyrin III structure

Coproporphyrins I–IV are isomeric tetrapyrroles which have four propionate side chains attached to the periphery of the macrocycle (Fig. 4B, inset). The isomers differ in the positions of the propionates. To confirm the conclusion drawn from the mass spectrometric data and to identify the coproporphyrin isomer(s) present in our sample, the red pigment labelled with ^{13}C and ^{15}N was studied with a battery of NMR experiments. In the interpretation of the spectra, the published NMR spectra of coproporphyrins [12,15,16] and their derivatives were utilized.

In general, the NMR data agreed with the mass spectrometric analyses, suggesting a coproporphyrin structure for the red pigment. Among the measured chemical shifts in ^1H – ^{13}C heteronuclear single quantum coherence (HSQC) spectroscopy (Table 1), three special spectral features were observed: first, the aliphatic region of the HSQC spectrum contained two sets of signals near 3 ppm and at 4.3 ppm (Fig. 4A). These represent the chemical shifts of β - and α -methylenes of the propionates. Upon addition of zinc acetate to the sample, the minor signals disappeared and the spectrum simplified (Fig. 4B). A likely explanation for this behaviour is that the minor signals arose from a ca. 10% population of metal-free coproporphyrin III in the sample. Second, the four meso protons of the porphyrin ring appeared to give rise to three NMR signals: 10.34 ppm (one proton), 10.06 ppm

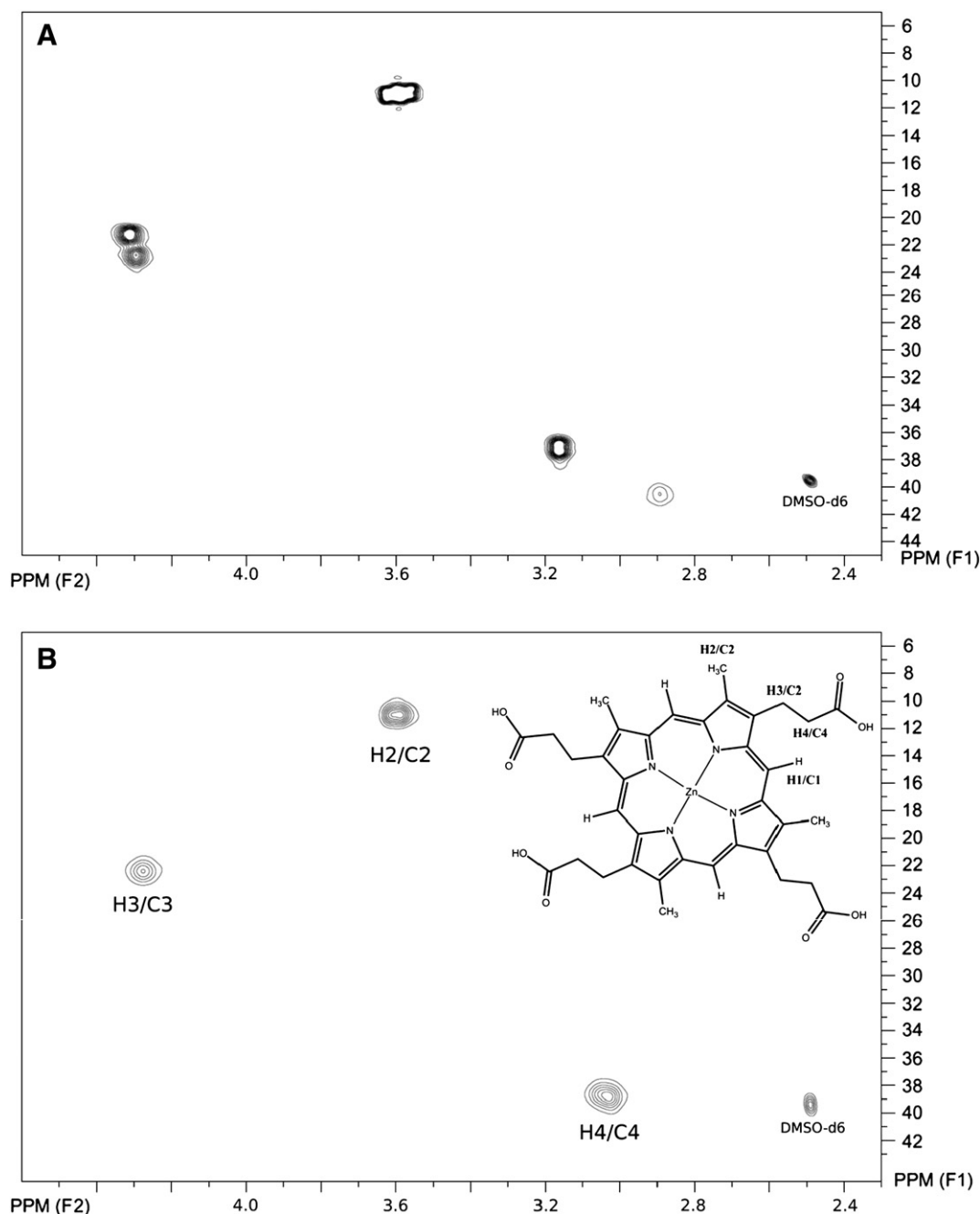


Fig. 4. Comparison of the aliphatic region of the ^1H – ^{13}C HSQC NMR spectra of the $^{13}\text{C}/^{15}\text{N}$ labeled red pigment before (A) and after (B) addition of zinc acetate. x- and y-axes refer to the ^1H and ^{13}C resonances in ppms, respectively. The addition of Zn^{2+} eliminates the heterogeneity of the signals near 3, 3.6 and 4.3 ppm for protons, suggesting that the heterogeneity reflects the presence of about 10% population of metal-free coproporphyrin III in the sample. The inset shows the inferred structure of the red pigment, i.e. that of zinc coproporphyrin III. For assignments of the signals, see Table 1.

Table 1

NMR chemical shift assignments from HSQC spectroscopy of the red pigment. The labeling of the protons and carbons refers to the inset in Fig. 4B.

^1H chemical shift	^{13}C chemical shift	Notes	Interpretation (cf. Fig. 4)	Comment
3.2 ppm	37.3 ppm	Major (proton signal shifts to 3.05 ppm upon addition of Zn^{2+})	Aliphatic CH_2 H4 and C4 in β -methylene of the propionate side chain of zinc coproporphyrin III	
2.9 ppm	40.5 ppm	Minor (ca. 10%; signals disappear upon addition of Zn^{2+})	H4 and C4 of free coproporphyrin III	
3.6 ppm; (two singlets)	11.0 ppm		Aliphatic CH_3 H2 and C2 of the β -methyl attached to the porphyrin ring	Proton signals diagnostic of coproporphyrin III structure
4.3 ppm	21.3 ppm	Major	Aliphatic CH_2 H3 and C3 in α -methylene of the propionate side chain of zinc coproporphyrin III	
4.3 ppm	22.8 ppm	Minor (ca. 10%; disappears upon addition of Zn^{2+})	H3 and C3 of free coproporphyrin III	
10.34 ppm 10.06 ppm 10.02 ppm (3 broad singlets)	96.4 ppm		Porphyrin ring CH H1 and C1 at the meso position of zinc coproporphyrin III	Proton signals indicate coproporphyrin III isomer

and 10.02 ppm (three protons integrated together, see Fig. 5). Third, the signal of the β -methyls at 3.6 ppm was divided into two broad peaks in proton NMR. The latter two observations identified the red molecule as the coproporphyrin III isomer. The other NMR signals, although not conclusive regarding the isomer type, were consistent with this interpretation.

Finally, a TLC experiment showed that the purified red pigment migrated identically with a zinc coproporphyrin III standard (data not shown). The migration of zinc coproporphyrin I standard was clearly slower, as also observed before [10,11]. Taken together, the above optical, fluorescence and NMR spectroscopy as well as mass spectrometry and TLC experiments strongly suggest that the secreted red pigment consisted predominantly of zinc coproporphyrin III.

4. Discussion

Our data suggests that copper deprivation brings about secretion of zinc coproporphyrin III to the growth medium of *P. denitrificans*. As

shown in Fig. 1, the production of this red pigment reached its maximum in the late logarithmic–early stationary growth phase. In some experiments, the concentration of the pigment was 5 μM . It appears that coproporphyrin production begins and copper limitation sets in after one or two days when the culture is approaching a high cell density. One should note that the inoculum was grown in normal LB medium, which normally contains enough copper to allow strong growth. It is therefore likely that residual copper originating from the starter culture had to be used up first until a copper-deficiency was established, leading to the observed production of coproporphyrin in the late log and stationary phases. The cell yield of copper-deficient cultures was typically 70–80% of the yield of control cultures, an indication that the cultures were suffering from copper limitation. Moreover, inclusion of 1 μM copper coproporphyrin in the copperless culture medium increased the cell yield to 90–95% of the control level. Apart from showing that the reduced growth yield was caused by lack of copper, this complementation experiment also implied that the microbe could use Cu-porphyrin as a source of copper.

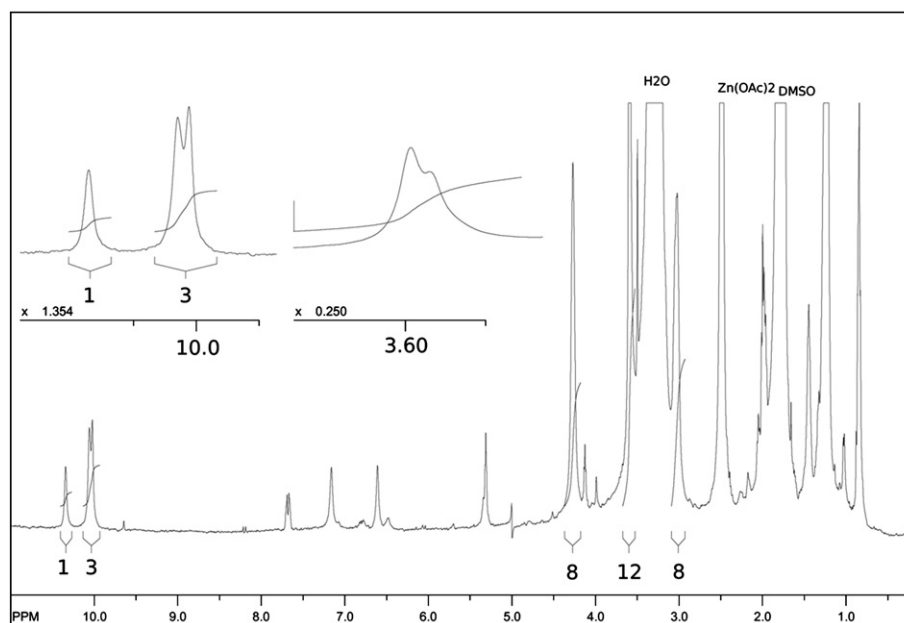


Fig. 5. A ^{13}C -decoupled ^1H NMR spectrum of the red pigment after addition of zinc acetate. The three signals of the meso protons at 10.24, 10.06 and 10.02 ppm and the proton signal of the β -methyls at 3.6 ppm are shown in detail, as they are diagnostic of the coproporphyrin III structure. The numbers beneath the signals at 10 ppm, 4.3 ppm (propionate aliphatic CH_2), 3.6 ppm and 3.2 ppm (propionate aliphatic CH_2) indicate the number of protons giving rise to each of the signals. H_2O , $\text{Zn}(\text{OAc})_2$ and DMSO refer to the resonances brought about by water, added zinc acetate and the solvent dimethyl sulfoxide. Ppm values on the x-axis indicate the proton chemical shift.

Intracellular coproporphyrin III is an apparent dead end side product of heme and chlorophyll biosyntheses and is therefore the most common of the four coproporphyrin isomers known [17–19]. Extracellular zinc coproporphyrins I and III have been found in meconium, i.e., in the sterile first stool of a newborn [12]. However, the biological function of coproporphyrin III is poorly understood. Coproporphyrin isomers and their relative amounts in urine and feces have been used as diagnostic markers for different porphyria types [18]. In particular, coproporphyrin III can accumulate if the enzyme coproporphyrinogen oxidase does not function properly. Yet it is unlikely that a poorly operating coproporphyrinogen oxidase would be the underlying reason for the overproduction of coproporphyrin III reported here. Although one could speculate that coproporphyrinogen oxidase activity requires copper and that copper deficiency therefore would lead to coproporphyrin accumulation, this does not agree with currently known properties of coproporphyrinogen oxidases [19,20]. Yet, owing to scarce information about terminal heme biosynthetic enzymes in prokaryotes, “a bacterial porphyria”, induced by lack of copper, cannot be fully excluded as a possible reason for the observed coproporphyrin production.

In bacteria, zinc coproporphyrin III has been identified in the culture medium of *Streptomyces* sp, also a soil-borne microbe like *P. denitrificans* [21]. *Bacillus cereus* grown in the presence of relatively high copper concentration (40–100 μM) was reported to accumulate intracellular copper coproporphyrin III [22,23]. Yamada-Ankei et al. [23] also studied the effect of other heavy metals (e.g., Mn, Zn, Co and Fe) in the culture medium on coproporphyrin production. In their study, all other metals failed to become chelated by the porphyrin, suggesting that copper porphyrin forms specifically in a controlled intracellular milieu. Our growth medium contained 90 μM Fe^{3+} , 50 μM Mn^{2+} and 10 μM Co^{2+} , all of which can form highly stable metalloporphyrins (see, e.g., [14]). Yet none of these was detected, suggesting that the binding of zinc kinetically favoured in the absence of copper. In contrast, on the basis of the observed two fluorescence emission maxima (the minor one at 615 nm representing the metal-free and the major one at 575 nm the zinc-bound porphyrin [12]), a small population of free coproporphyrin appeared to be present in the stationary phase culture media. NMR spectroscopy also suggested that about 10% of the total coproporphyrin was free and not complexed with zinc. Moreover, 10–15% of the red pigment in the culture medium bound tightly to the copper column (Section 3.2). Although optical spectra (Fig. 2) indicated that both the strongly and weakly binding fractions contained mixtures of copper and zinc coproporphyrin, mass spectrometry suggested that the tightly bound form was copper coproporphyrin, while the loosely bound fraction contained mostly zinc as the central atom. The strongly binding fraction might have formed by copper binding during the chromatography, thus reflecting the presence of metal-free coproporphyrin in the spent culture medium. The weakly binding fraction was predominantly zinc coproporphyrin from the spent culture medium. Typically, the weakly binding fraction also yielded some fractions with the mass of 715 in LC/MS. These might be the result of a relatively slow $\text{Zn(II)} \rightarrow \text{Cu(II)}$ metal exchange reaction taking place during the chromatography, an possible reaction owing to the higher stability of the copper tetrapyrrole [14].

The only copper-specific chalkophore known so far is methanobactin ($M_r = 1215$), a complex peptide isolated from the growth media of two methanotrophic soil bacteria [4,5]. These bacteria live under anaerobic conditions utilising methane gas generated by other microbes in the lower soil layers. Copper is an important micronutrient to the methanotrophs, because it comprises the active site of the enzyme particulate methane mono-oxygenase [24]. Under the prevailing poorly aerated soil conditions, copper may exist as a cuprous ion, Cu^+ , which is much less water-soluble than Cu^{2+} . The function of methanobactin may therefore be not only to scavenge copper but also to render it soluble for the bacterial cell. It is thought that methanobactin preferentially binds Cu^+ so that also bound Cu^{2+} is subsequently reduced to Cu^+ [4].

Our results suggest that copper-poor conditions induce the excretion of zinc coproporphyrin III to the medium and that this molecule could play the role of a chalkophore in *Paracoccus*. It is possible that the formation of zinc coproporphyrin resulted from our experimental conditions (with 25 μM Zn^{2+} in the growth media) and that the actual physiological chalkophore was the metal-free porphyrin, observed with fluorometry in the spent culture media and as the tightly-binding fraction in immobilised copper chromatography and as a minor component in the NMR measurements (Fig. 4).

Like the methanotrophs, *P. denitrificans* is a soil bacterium [6]. However, in spite of being able to denitrify under anaerobic conditions, *Paracoccus* can also live fully aerobically [6]. Conceivably, the habitat of *Paracoccus* is typically much less anaerobic than that of *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b. This means that copper would exist mainly as Cu^{2+} , for which free coproporphyrin III has a very high affinity [14,25]. In fact, coproporphyrins are relatively specific for copper, so that they have been used in analytical applications for copper determinations of biological water specimens containing submicromolar $[\text{Cu}^{2+}]$ together with other metal ions [25–29]. The main problem in the analytical use of coproporphyrins as copper chelators appears to be the slow (on the time scale of minutes) formation of the complex¹, which can be speeded up by a number of mechanisms: metal exchange (e.g., replacement of zinc with copper as the porphyrin central atom), presence of bases such as imidazole or pyridine or by inclusion of a reductant. As discussed above, copper coproporphyrin is significantly more stable than zinc coproporphyrin and, in our hands, metal exchange may have occurred during immobilized metal affinity chromatography. A reductant such as ascorbate has been found to catalyze the formation of a copper porphyrin [28,29]. Ascorbate is likely to convert the well solvated Cu^{2+} to the poorly solvated Cu^+ , which however appears to be kinetically more capable to bind to the porphyrin.

Subsequently, the bound Cu^+ may reoxidize back to Cu^{2+} . Marginally soluble Cu^+ could form under anaerobic soil conditions and the capacity to bind both cupric and cuprous ions might thus be biologically relevant even for *P. denitrificans*.

It is also possible that under our copper-poor conditions a problem could have arisen because too much zinc relative to copper was present and that the red pigment would have been a detoxification mechanism to lower the amount of free zinc. For this reason, we studied the effect of reducing the amount of zinc in the culture media. Even when the concentration of added zinc in the growth media was as low as 1 μM , the red pigment was observed in copper-poor cultures (not shown). Regardless of the Zn concentration, the amount of zinc in the pigment fraction was maximally about 20% of the total added zinc, leaving most of the metal not bound to the porphyrin. Therefore we consider it unlikely that the function of the pigment would be to reduce the concentration of free zinc under copper-poor conditions. Rather, it is possible that the zinc porphyrin is a stable side product remaining in solution after a fast reaction of copper and the free porphyrin with subsequent uptake of the copper complex into the cell. It is known that copper reacts with porphyrins about ten times faster than zinc [29]. Finally, it is noteworthy that abundant free and copper porphyrins have been found in soil samples [30]. The authors proposed that these porphyrins originated in plants and algae and participated in fixation of copper. However, our study opens up the possibility that the soil porphyrins could also be of microbial origin and that they are in fact bacterial copper-acquisition compounds.

If *P. denitrificans* uses coproporphyrin III for copper scavenging, it should also possess a system for uptake of the metallated tetrapyrrole (or a system for extracting copper bound to the porphyrin). Analysis of the available *P. denitrificans* genome shows the presence of a TonB-

¹ This relatively slow kinetics may not be a serious problem under biological conditions. Cu(II) and to a lesser extent Zn(II) are the fastest to be chelated nonenzymically by an aqueous porphyrin [29,31].

dependent heme receptor/transporter [32,33], which could be involved in the uptake of copper-coproporphyrin complex. Intriguingly, the gene for this receptor (locus tag Pden_4201) is situated near the locus coding for the multicopper enzyme nitrous oxide reductase (Pden_4219) and its assembly factors. TonB-directed heme transport normally brings the captured tetrapyrrole through both outer and inner membranes to the cytoplasm. A periplasmic binding protein together with an ABC transporter may catalyze the passage of the metallated tetrapyrrole through the inner membrane. Since the biosynthesis of many copper proteins such as nitrous oxide reductase takes place in the periplasm, copper transported to the cytoplasm would need first to be released from its chelator and then translocated 'backwards' to the periplasm. A very recent report suggests that nitrous oxide reductase biosynthesis in *Rubrivivax gelatinosus* requires a P-type copper-translocating ATPase, which was proposed to operate in this direction, i.e., to transport copper from the cytoplasm to the periplasm [34]. The *Paracoccus* genome carries several genes coding for putative P-type Cu-ATPases, similar to those discovered by Solioz et al. [35]. In addition, the genome of *P. denitrificans* contains a homologue of YcnJ, encoding a putative inner membrane copper transporter in the gram-positive *Bacillus subtilis* [36] (which does not have the TonB-system of gram-negative bacteria). Several other players (e.g., NosD and NosL), thought to have specific roles in copper-acquisition for nitrous oxide reductase in *Pseudomonas stutzeri* [37], are also present *P. denitrificans*. Interestingly, however, the *Paracoccus* genome appears to lack NosA/ OprC, a poorly understood TonB-dependent copper transporter in the outer membrane of several pseudomonads. This suggests that another copper-acquisition system is in use at the *Paracoccus* outer membrane. Even in *Pseudomonas stutzeri*, gene deletion mutagenesis showed that the OprC locus is not indispensable for synthesis of active nitrous oxide reductase under copper limitation [37], implying that another system was used for copper-acquisition. All pseudomonads contain several members of the TonB-dependent transporter family and, for example in *Ps. aeruginosa*, the gene for hasR-heme uptake receptor is located close to the gene for nitrous oxide reductase.

In conclusion, an attractive hypothesis is that, under copper-limited conditions, a TonB-dependent copper tetrapyrrole uptake system would operate in *Paracoccus* and perhaps also in pseudomonads active in nitrous oxide reduction.

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